APPLICATION FOR UNITED STATES LETTERS PATENT
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Title: METHOD OF AMPLIFICATION FOR INCREASING THE SENSITIVITY OF DETECTING NUCLEIC ACID-PROBE TARGET HYBRIDS

Inventors: Eng Jom Aw
22 Fernbrook Place
Kanata, Ontario K2M 2L8
CANADA

A Citizen of Canada

Sithian Pandian
6100 Voyageur Drive
Orleans, Ontario K1C 2P6
CANADA

A Citizen of Canada

David I. Smith
4 Lulworth Court
Richmond, Ontario KOA 2Z0
CANADA

A Citizen of Canada

BACKGROUND

The present invention relates to nucleic acid hybridization assays which are useful as a means of locating specific nucleic acid sequences. Examples of nucleic acid sequences are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) sequences. The molecular subunits of both DNA and RNA are called nucleotides which are linked together to form long polynucleotide chains. Each nucleotide subunit is made of a sugar moiety, a phosphate moiety and a base moiety. It is the sequential ordering of the base moieties [adenine (A), cytosine (C), guanine (G), thymine (T), uracil (U)] that contains DNA or RNA's genetic information. The ordering of these base moieties in a polynucleotide chain and the tendency of the bases to attract and bond with other specific base moieties, is exploited by this invention to locate, detect and isolate specific DNA or RNA sequences.

DNA normally contains two polynucleotide strands twisted about one another lengthwise in a helical manner resembling a ladder where the sides are made of identical sugar (deoxyribose) and phosphate molecules while the rungs are made up of bases extending out from each strand, held together by weak attractive forces. In DNA, the base thymine on one strand always pairs with the base adenine on the opposing strand, and the base guanine always pairs with the base cytosine. This is called complementary base pairing.

RNA is also a polynucleotide strand. However, the sugar moiety is ribose (versus deoxyribose in DNA) and the bases are adenine, guanine, cytosine and uracil. In RNA, the base uracil on one strand always pairs with the base adenine on the opposing strand, and the base guanine always pairs with the base cytosine. Although RNA can pair with either a complementary strand of RNA or DNA, it is normally single stranded so does not form a helical structure.

The present invention is founded, in part, upon the technique that single stranded nucleic acid sequences can be combined, or hybridized, under appropriate conditions with complementary single stranded nucleic acid sequences to form double stranded molecules. This technique was developed as a means for detecting and/or and isolating particular nucleic acid sequences of interest. It has increased in popularity during recent years in its application for detecting the presence of the DNA or RNA within such pathogens as viruses, bacteria, or other microorganisms and therefore the presence of these pathogens themselves. The technique can also be used for other purposes such as to screen bacteria for antibiotic resistance, to aid in the diagnosis of genetic disorders (for example in sickle cell anaemia and thalassaemia), and to detect cancerous cells. Several applications have been developed for the microbiological analysis of clinical, food, environmental, and forensic samples. A general review of the technique and its present and future significance is provided in Biotechnology (August 1983), pp. 471-478 which is incorporated herein by reference.

The following definitions are provided to facilitate an understanding of the present invention. The term 'probe' refers to a nucleic acid sequence of which there are at least three types: the primary probe, the amplification probe, and the labelling probe. The primary probe contains at least one nucleic acid sequence that is complementary (or will base pair) to some portion of a nucleic acid sequence on the target DNA or RNA molecule of interest. The amplification probe contains sequences that are complimentary to some sequences on the primary probe, and contains a region that is typically of at least one type of repeating sequence unit. The labelling probe contains sequences complementary to one of the repeating sequence units, in addition to a chemical label. Labels are detectable chemical groups, either radioactive molecules or non-radioactive molecules and can include: radioactive isotopes; enzymatically active groups such as horse radish peroxidase; fluorescent agents; chemiluminescent agents; precipitating agents; and/or dyes. The term 'signal' is

used loosely to indicate the detectable characteristic of a detectable chemical group, which can include: a change in the light adsorption characteristics of a reaction solution resulting from enzymatic action of an enzyme attached to a labelling probe

5   acting on a substrate; the color or change in color of a dye; fluorescence; phosphorescence; radioactivity; or any other indicia that will be evident to one skilled in the art.

The amplification probe is so named because it is used to cause many detectable chemical labels to become attached to one

10   probe-target complex, such that the resulting signal is amplified in direct proportion to the number of labelled probes that hybridize to the amplification probe. If the amplification probe were to contain only one sequence unit that comprises sequences compatible to the labelling probe, only one labelling probe would

15   become attached to the probe-target complex, and the signal would not be amplified. However, the amplification probe disclosed in the present invention contains typically five or more sequence units (also referred to as repeatmers) that are combatable to the labelling probe, such that five labelling probes will attach to

20   one probe-target complex, resulting in five times the amount of detectable chemical label signalling the presence of one probe-target complex; thus, the indication that one probe-target complex was formed will be amplified five times. Moreover, if the amplification probe contains sixteen sequence units that are

25   combatable to the labelling probe, sixteen labelling probes will attach to one probe-target complex, resulting in sixteen times the amount of detectable chemical label signalling the presence of one probe-target complex; the indication that one probe-target complex was formed will thereby be amplified sixteen times. The

30   degree of amplification is optional and can be manipulated by the design and construction of the amplification probe as described herein.

One objective of a nucleic acid hybridization assay is to detect the presence of a specific nucleic acid sequence (the

35   target sequence) in a given sample by contacting the sample with

a complementary nucleic acid sequence (the probe) under hybridising conditions and observing the formation or absence of any probe-target complexes. The probe-target complex can be detected directly by a label attached to the probe. The complex

5   can also be detected indirectly through such techniques as the hybridization of another nucleic acid sequence conjugated to a label or by the binding of an antibody labelled with a detectable chemical group.

One detection strategy currently employed in the art is

10   exemplified by PCT Application 84/03520 and EPA 124221 which use an enzyme labelled nucleic acid sequence to detect the probe-target complex by hybridization to complementary sequences on the tail of the probe. For example, the Enzo Biochem "Bio-Bridge" system uses a biotin molecule conjugated to a poly(A) tail (a

15   nucleic acid sequence comprised solely of adenine nucleotides) as the detection system following hybridization of a DNA probe possessing a poly(T) tail (a nucleic acid sequence comprised solely of thymine nucleotides) to the target DNA sequence.

In order to employ such a technique as an assay, one must

20   be able to detect the presence or absence of probe-target complexes with a high degree of sensitivity. The sensitivity of a nucleic acid hybridization assay is determined primarily by the detection limit of the label to demonstrate the formation of the probe-target complex against back-ground noise and/or false-

25   positives. Different strategies have been employed to improve the sensitivity of nucleic acid hybridization assays, which can be classified into four broad categories: 1) separation of the probe-target complex; 2) target amplification; 3) probe amplification; 4) multiple labelling, or combinations thereof.

30   Some nucleic acid hybridization assays involve immobilization of the target sequence on a solid support followed by washing away the remainder of the reaction mixture. This first category involves techniques that attempt to either immobilize the target sequence before adding a label probe or use

35   an immobilized labelled probe to capture the target nucleotide

sequence. Alternatively, techniques have been developed that immobilize the probe-target complex after its formation. For example, EPA Publication No. 0225807 discloses a nucleic acid hybridization assay in which the probe-target complex is removed from solution by hybridization with a complementary solid-supported capture probe. The solid phase complex is then detected by subsequent hybridization to a labelled probe. Generally, procedures attempting to immobilize the probe-target complex at this stage using a nucleic acid sequence suffer from the fact that proteins and other materials in the heterogeneous sample may have a higher tendancy to interfere with the immobilization of the nucleic acids. Furthermore, the sensitivity is low as the label to target ratio is 1:1.

A second category of strategies involves increasing the sensitivity of a nucleic acid hybridization assay through target amplification. An example of target amplification entails assaying for ribosomal RNA (rRNA) of a microorganism rather than chromosomal DNA. Since rRNA is present in any given cell at 10^4 times higher concentration than DNA, the number of possible probe-target complexes increases, thereby increasing the probability of detecting the target organism. Alternatively, the polymerase chain reaction (PCR™) described in United States patents 4,683,105 and 4,683,202 has been used to amplify target nucleic acid sequences. The advantages and limitations of this technique has been reviewed by Gyllensten (Biotechniques 7, 700-706, 1989, incorporated herein by reference). For example, this transcription-based amplification system can produce a 2-5 million-fold amplification of a RNA target after 4 cycles (Lizardi et al., Biotechnology 6, 1197-1202, 1988). However, this technique suffers from such problems as excessive noise, false positives, requires considerable technical expertise, and relatively expensive instruments and reagents (Walcott et al. Food Protein 54:387-401, 1991).

A third category of strategies for increasing the sensitivity of a nucleic acid hybridization assay entails probe

amplification, by employing a combination of primary probes. Examples of this method are disclosed in United States patents 4,731,325 and 4,868,105 wherein techniques describe the use of more than one probe that binds to the target nucleic acid

5   sequence. A further example is found in U.S. Patent 4,868,105, where the labelled secondary probes hybridizes to the multiple primary probes bound to the target nucleic acid sequence.

Finally, some have attempted to employ multiple probes in a cascading or sandwich fashion as a strategy for amplifying the

10   signal. These methods fall under the fourth category of signal amplification because they result in the attachment of multiple labelling groups to the primary probe-target complex. It is within this category that the present invention could be said to reside.

15   An early attempt to develop strategies within this fourth category is exemplified by PCT Application WO 90/13667 which describes an amplified solution-phase sandwich nucleic acid hybridization assay for the hepatitis B virus nucleic acid sequence in which the analyte is hybridized in solution with sets

20   of amplifier probe polynucleotides and capture probe polynucleotides each have a first segment that is complementary to the target nucleic acid; furthermore, the amplifier probe has a second segment that is complementary to a unit of a polynucleotide multimer whereas the capture probe has a second

25   segment that is complimentary to a polynucleotide bound to a solid phase. The resulting product is reacted with the polynucleotide bound to a solid phase and then with the multimer. The multimer probe is a chemically cross-linked single stranded oligodeoxyribonucleotide star-structured complex with arms

30   possessing sequences complementary to the primary probe. Detection occurs when the bound materials are reacted with a labelled probe complementary to the polynucleotide units of the multimer.

In spite of the limited use of this strategy for detecting hepatitis B virus, the difficulties one would face devising the assay reagents are manifold, especially for general use. Constructing the chemical cross-linking in the secondary probe

5 involves high levels of technical expertise as well as careful chemical modification of these polynucleotides in order to bring about the desired cross-linking. Overloading the star-like structures of the secondary probe can lead to steric hindrance between the star-like structures as well as between the anchoring

10 arms attached to the solid phase. Furthermore, two sets of primary probes, both requiring the sequences complementary to the target nucleic acid, demand synthesis of probes designated to only one given target which is the case for a consensus hepatitis B virus double-stranded region sequence based on a multiplicity

15 of hepatitis B viral subtypes. With these methods the cost of preparing probe reagents becomes significantly elevated eventually reflecting in the overall cost of the assay or the diagnostic kit. In short, this strategy is technically complex and the probe reagents are not applicable to a large variety of

20 targets. Therefore, there remains a general need for a simpler system of signal amplification.

In yet a further method, Canadian Patent Application 2,039,517 provides a method for amplifying a signal wherein the amplification is obtained through use of a bridging nucleic acid

25 sequence which can hybridize to the primary probe and to a developer nucleic acid sequence. This method entails hybridizing the primary probe to the target sequence, followed by exposure to the bridging sequence, followed by exposure to a first developer molecule, and finally followed by a second developer

30 molecule to form a developer chain. One of the developer molecules is labelled, and the labelling can be detected in the developer chain. Again, the major limitation of this strategy is its complexity and there still remains a need for a simple system that allows for an increase in sensitivity.

A further method for this category is found in United States Patent 4,716,106 where the primary probe sequence is first cloned in the filamentous phage M13 DNA. The single stranded form of the M13 DNA carrying the target-complementary sequence is isolated and then used as a primary probe. The DNA strand complementary to that carrying the probe is also separately isolated, labelled at multiple sites along its length and then used as the detector probe. Even though this assay involves the use of multiple labelling, this strategy necessarily involves cloning of the probe sequences in the M13 phage. Given the present molecular biology methodology, cloning of M13 is a difficult and cumbersome process, even for someone skilled in the art. The cloning must be performed every time anew in order to prepare the probe reagents directed towards a given target nucleic acid sequence. This increases the time, effort, and cost involved. Therefore, there remains a need for a simpe and sensitive assay system for detection of specific nucleic acid sequences.

Due to the complexity and involvement of each of the strategies described above, these techniques are used to a limited extent by laboratories. Therefore, a need continues to exist for a simplified, rapid, and adaptable hybridization assay wherein a primary probe hybridizes to a target and to an amplifier polynucleotide strand that allows for attachment of multiple copies of a labelling molecule.

The present invention serves to overcome the limitations of the assays currently available in the art. Those ordinarily skilled in the art will appreciate that a preferred embodiment of the present invention provides a method for amplifying a signal during the detection of target nucleic acid sequences comprising:

    a)   hybridizing a polynucleotide probe (the primary probe) to the target nucleic acid sequence wherein the primary probe has a means for binding to a

amplification nucleic acid sequence (the amplification probe), the amplifying molecule being capable of hybridizing to at least one labelling nucleic acid sequence, conjugated to a chemical label (the labelling probe);

b)   immobilizing the probe-target complex;

c)   exposing the immobilized probe-target complex to an amplification probe (a polynucleotide sequence containing a region of multiple repeating sequence units and a region of sequences complementary to sequences in a region of the primary probe) under conditions that allow the amplification probe to hybridize to the probe-target complex;

d)   exposing the hybridized amplification probe to a labelling probe (a nucleic acid sequence conjugated to a chemical label and containing a region of sequences complementary to a repeating sequence unit on the amplification probe) under conditions that allow many labelling probes to hybridize to the amplification probe;

e)   detecting the labelling probes in the resulting complex.

There are many advantages over the prior art attained by this invention. First and foremost, the present invention greatly facilitates label detection in nucleic acid hybridization assays by providing a plurality of repeating units in the amplification probe whereby it may receive up to sixteen molecules (or more) of a selected labelling probe for every probe-target complex. This results in significantly increased sensitivity, and enhanced utility of hybridization assays.

A further advantage resides in the design of the probe used
in the cascade of reactions. These probes could be easily and
economically synthesized with the currently available methods and
instruments. The rate of hybridization reaction is much higher
in this invention due to the use of many shorter probes than when
longer probes are used. Moreover, use of single stranded probes
also avoids the problem of self-annealing of the probes during
the hybridization reaction. Since the label is not directly
linked to the primary probe, the hybridization properties of
primary probe are not altered, which contributes to the ability
of probe reagents to be applicable in a large number of target
detection assays. Therefore, the preparation of such reagents
is achieved by simple methods, reducing the overall cost of the
assay.

Yet a further advantage is seen in the fact that since the
primary probe could always be synthesized with a poly(A)
sequence, the other components used in the assay such as the
amplification probe and the labelling probe could be used
universally in any nucleic acid hybridization assay. This
translates into a large economy of efforts, costs, and uniformity
of reaction conditions. Further, this allows the possibility for
quickly developing assays for any given target; the only new
requirement for each project would be the construction of the
specific portion of the primary probe.

A further advantage of the present invention is to provide
hybridization assays having less background noise, greater
sensitivity, higher signal/noise ratios, as well as greater speed
than has been achievable with previously known methods.
Traditionally in nucleic acid hybridization assays, the primary
probe is labelled with biotin or other markers along its entire
length. When required to expedite the hybridization reaction
using polynucleotides, such extensive modification of the probe
might lead to a reduced efficiency of its hybridization with the
target molecule and therefore the probe-target complexes might

not be formed rapidly or stably. Since the primary probes of these traditional assays are end labelled, each of the probe-target complexes would carry only one marker and therefore the sensitivity of the assay would be limited to the detection of one
5   marker per probe-target complex. In contrast, one embodiment of the present invention would amplify the signal by adding sixteen or more marker molecules per each probe-target complex. The sensitivity of the assay would therefore be amplified to the extent proportional to the number of marker molecules attached
10   to the amplification probe.

In a preferred embodiment of the present invention, a method is provided for amplifying a signal during the detection of target nucleic acid sequences in a test sample containing cellular material comprising the steps of hybridizing a
15   polynucleotide probe (the primary probe) to the target nucleic acid sequence wherein the primary probe has a means for binding to a amplification nucleic acid sequence (the amplification probe), the amplifying molecule being capable of hybridizing to at least one labelling nucleic acid sequence, conjugated to a
20   chemical label (the labelling probe); immobilizing the probe-target complex; exposing the immobilized probe-target complex to an amplification probe (a polynucleotide sequence containing a region of multiple repeating sequence units and a region of sequences complementary to sequences in a region of the primary
25   probe) under conditions that allow the amplification probe to hybridize to the probe-target complex; exposing the hybridized amplification probe to a labelling probe (a nucleic acid sequence conjugated to a chemical label and containing a region of sequences complementary to a repeating sequence unit on the
30   amplification probe) under conditions that allow many labelling probes to hybridize to the amplification probe; detecting the labelling probes in the resulting complex.

In another embodiment, cells to be assayed are lysed with a lysis buffer solution and the nucleic acid sequences are
35   denatured. The primary probe is contacted with the denatured

nucleic acids in solution phase. Any probe-target complexes formed are removed from other material by immunocapture of the complexes. This is achieved by transferring the hybridization reaction mixture to microtiter plate wells that have been previously coated with monoclonal anti-probe-target complex. After washing, the immobilized probe-target complex is detected by contacting it with the amplification probe, then the labelling probe. The presence of an enzyme attached to the immobilized probe-target complex is determined by contacting the enzyme with its substrate and measuring the resulting reaction. Such an embodiment can involve the detection of *Listeria monocytogenes* in cheese, the detection of *Escherichia* coli in meat, or the detection of *Salmonella typhi* in human blood.

FIGURES

Certain embodiments of the invention may be seen from the Figures.

Figure 1 is a schematic representation of the construction of the plasmid preparation for reproducing the amplification probe.

Figure 2 depicts an embodiment wherein multiple probes are used in a sequential fashion in an assay for detecting a target nucleic acid sequence. In step I, the cellular material is lysed, the nucleic acids are denatured and a specific synthetic DNA probe is added. Step II comprises hybridizing the specific DNA probe to the target nucleic acid within the sample to generate heteroduplex nucleic acid (HNA) complexes. The HNA complexes are immunocaptured by antibodies attached to a solid support such as a microtiter plate as depicted in Step III. Steps IV and V depict the series of steps involved in detecting the immunocaptured HNA complexes. In step IV, riboKAPROBE is added and allowed to incubate for 30 minutes to enable hybridization with the 3' tail of the probe. Step V depicts: i) addition of KAR-25AP followed by a 30 minute incubation step; and ii) addition of substrate and subsequent incubation for 5 to 30 minutes. The developed color was then detected by conventional means.

DETAILED DESCRIPTION OF THE INVENTION

This invention comprises the preparation of a number of nucleic acids sequence probes. This invention also comprises a method of simplifying and significantly amplifying the signal generated in a nucleic acid hybridization assay. The simplification and amplification is primarily achieved by

using an amplification probe comprising multiple repeating sequence units and labelling probes that are complementary to the repeating sequence units.

The Target Nucleic Acid Sequence

5   The target nucleic acid sequences that can be detected in accordance with the present invention may be any nucleic acid sequence. There is no maximum limit to the length of the target nucleic acid sequence, though the minimum should be at least sixteen nucleotide bases in length.

10

The Primary Probe

The primary probe, which is single stranded nucleic acid sequence, has two distinct regions. At the 5' end, the
15   sequence is complementary to a sequence found in the target, and such sequence is of sufficient length, ranging from at least 6 nucleotides up to a maximum of any length desired. In a preferred embodiment the length ranges from 16 to 25 nucleotides. The 3' end of this primary probe comprises a
20   homopolymeric nucleotide tail [for example, a poly(dA)]. This poly(dA) sequence ranges in length, but must be sufficient to hybridize with the amplification probe. In a preferred embodiment the length ranges from 12 to 20 nucleotides.

When the target is DNA, the probe is preferably prepared
25   in the form of RNA. When the target is RNA, the probe is preferably prepared in the form of DNA. When hybridization of the probe to the target results in an RNA-DNA hybrid, this is known as a heteroduplex. In a preferred embodiment, the homopolymeric region of the primary probe remains DNA in
30   nature, though in the general embodiment, any type of nucleic acid sequence can be utilized in this region.

Techniques for synthesising a single-stranded polynucleotide sequence for the primary probe, which is

complementary to the target sequence, are well known in the
art and will not be described here.

The Antibody Reagent

Immobilization of the probe-target complex is achieved by
5   using an antibody, attached to a surface, that binds to
double-stranded nucleic acid. By separating the probe-target
complex from the rest of the sample mixture, this procedure
results in improved sensitivity of the detection of the
target.

10   These particular antibodies are well known in the
literature (Fliss et al., Appl. Environ. Microbiol. 59:2608-
2705, 1993; Coultee et al., Anal. Biochem. 181:96-105, 1989;
US patent 5,200,313, all incorporated herein by reference) as
is the procedure for the coating of the antibody molecules to
15   a surface. Whole antibodies, antibody fragments,
polyfunctional antibody aggregates, or in general any
substance comprising one or more specific binding sites from
an antibody for the probe-target complex can be utilized as
described herein. Unless otherwise noted, it should be
20   understood that, the term antibody when used in both the
disclosure and the claims means whole antibodies and their
polyfunctional and/or fragmented forms as well. When the term
refers to a whole antibody, it may belong to any of the
classes and subclasses of known immunoglobulins (IgG, IgM,
25   etc.). It is also possible that a fragment of any such
antibody which retains specific binding affinity for the
hybridized probe can also be used, such as, the fragments of
IgF which are often referred to as Fab, F(ab'), and F(ab)$_2$.
Furthermore, aggregates, polymers, derivatives, and conjugates
30   of immunoglobulins and/or their fragments can also be utilized
where appropriate.

The antibody reagent's immunoglobulin source can be
procurred from any available techniques such as conventional

antiserum and monoclonal techniques. Antiserum can be procurred through well-known techniques involving the immunization of an animal, (such as mouse, rabbit, guinea pig or goat) with the appropriate immunogen. Furthermore, the
5   immunoglobulins can be obtained by somatic cell hybridization techniques, which would result in the formation of monoclonal antibodies.

The preparation of immunogens for stimulating antibodies specific for heteropolymeric (ie. DNA-RNA or RNA-DNA) probe-
10 target complexes can be achieved through a variety of techniques. For example, one can employ transcription of ϕX174 viron DNA with RNA polymerase (Nakazato, (1980) Biochem. 19:2835, incorporated herein by reference). The resulting probe-target complexes can be adsorbed to a methylated
15 protein, or they can be linked to a conventional immunologenic carrier material such as bovine serum albumin, before being injected into the desired host animal (Stollar, (1980) Meth. Enzymol. 70:70, incorporated herein by reference).

The most important property of any antibody raised
20 against such target-probe double stranded complexes is that the antibody will significantly discriminate in its binding properties between the duplexed form of the target-probe complex and single stranded nucleic acid sequences. Antibodies do not need to recognize specific sequences.
25 Rather, they recognize the general double-stranded characteristic of the probe-target complex. This is a critical feature of this invention that significantly reduces background noise and false positives that could result from hybridization of labelling probe to non-target single stranded
30 nucleic acid sequences in the sample.

It is preferred to use a solid support to which the antibody is attached or fixed. Attachment of the antibody can be achieved through either covalent or noncovalent bonds. The latter includes adsorption techniques that provide for a

suitably stable and strong attachment. The solid support can
take on a variety of shapes and compositions. These include
beads, microparticles, porous and impermeable strips and
membranes, as well as the interior surface of reaction vessels
5   such as test tubes and microtiter plates, etc. The techniques
for attaching a desired reaction partner to a selected solid
support are well known to one skilled in the art.

The Amplification Probe

The amplification probe is the principal feature of this
10 invention and serves to cause a plurality of detectable
chemical labels to become attached to each amplification
probe; in this way, the signal indicating the formation of one
probe-target complex is amplified in direct proportion to the
number of labelling probes that hybridize to the repeating
15 sequence units.

The amplification probe is a single stranded nucleic acid
sequence consisting of at least two regions. The first region
contains a short nucleotide sequence complementary to a
portion of the primary probe that permits the amplification
20 probe to hybridize with the primary probe that is part of the
immunocaptured probe-target complex. In one embodiment, this
first region is located on the 3' end and is comprised of a
homopolymeric tail [e.g., poly(dU)] of approximately 12 to 20
nucleotides, but this can be extended up to any length
25 desired.

The second region contains multiple repeating sequence
units, that form the basis by which the detection indicia will
be amplified when a labelling probe (containing sequences that
are complementary to a region within one sequence unit) is
30 hybridized to each of these repeating units. The number of
repeating units can vary from 2 to as many as can be

accommodated within a particular application of this
invention. The greater the degree of amplification required
for a given test system or the less sensitive the detection
means to be employed, the larger the number of repeating units
5   required. In one embodiment, the number of repeating units is
about sixteen.

The length of each unit can vary, dependant upon the
requirements of a particular application of this invention.
The minimum length of a repeating unit is about 16
10   nucleotides, though the best length is about 70-100
nucleotides. One critical factor to consider when designing
the length of each repeating unit is the steric hinderance
caused by the size of the detectable chemical label attached
to the labelling probe. For example, a relatively large
15   enzyme would require a greater degree of spacing than a small
dye molecule or radioisotope. Moreover, the labelling probe
should hybridize to only a portion of a repeating unit; the
remaining nucleotides within the unit that are not
complementary to the sequences of the labelling probe function
20   as spacers to position the detectable chemical labels apart
from one another. Furthermore, it will be understood that the
subunits in the tandem repeating nucleic acid fragment orient
in the same 5'→3' direction. In the example chosen in Figure
1, each repeating sequence unit is 69 nucleotides long.

25   The repeating sequence units can be identical, or there
can be more than one different sequence unit. For example, it
may be desirable to attach more than one type of detectable
chemical label to the amplification probe, whereby a
corresponding number of differently sequenced labelling probes
30   will be required. In this situation one would construct the
repeating sequence units with more than one type of sequence,
each sequence being complementary to the sequence a type of
labelling probe. For example, if it would be desirable for
three types of labelling probes (e.g., probe X, probe Y and
35   probe Z) to attach to the amplification strand, the

complementary repeating sequence units (unit x, unit y and
unit z) on the amplification probe could be constructed in a
xyzxyzxyzxyz fashion. The nucleic acid sequences that are
joined to form the repeating sequence region could each be
5   constructed in a xyz fashion, such that when they are joined
they will become linked in a xyz-xyz-xyz manner.

The construction of a new and unique plasmid that can be
used to produce multiple copies of the amplification probe is
prepared by well known techniques and one example is
10   illustrated in Figure 1. The DNA fragment coding for the
amplification probe is cloned in an appropriate plasmid, such
as pBluescript™, or any other suitable cloning vector. This
plasmid is used as a template for the preparation of the
amplification probe which consists of the complementary RNA
15   copies of the repeating units and the homopolymeric tail.

The DNA Vector and Cloning

The experimental methods used to construct the DNA
vectors used to reproduce the amplification probe are
generally described in various manuals of molecular biology
20   and are known to one who is skilled in the art (Sambrooke et
al., Molecular cloning: A Laboratory Manual, 2nd edition. Cold
Spring Harbor, N.Y., 1989, incorporated herein by reference).

In one instance, the vector plasmid pBluescript II SK+
(Stratagene, La Jolla, California) was linearized with the
25   restriction enzyme Bam H1. The following oligonucleotide
fragments were then self-annealed and ligated to the
linearized vector plasmid:

        Bam H1
        5' GATCC AAA AAA AAA AAA AAA AAA G 3'
30   3' G TTT TTT TTT TTT TTT TTT CCTAG 5'
                                      Bam H1

The annealed recombinant plasmic vector (pBat), obtained in the above ligation reaction, was introduced into *E. coli* (Epicurian E.coli XL-1; Stratagene, Inc.) using a bacterial transformation procedure recommended by Stratagene. Positive transformants were selected as white colonies and were further verified by colony hybridization in which denatured DNA of the colonies were hybridized with biotinylated poly A probe, followed by reaction with streptavidin-alkaline phosphatase and then with a chemiluminescent substrate. All the white colonies were positive to the poly A hybridization.

The plasmid pBat was then linearized with the restriction enzyme *Pst* 1 and ligated to the concatemerized repeatmers (containing *Pst* 1 site at one end and *Ase* 1 site at the other end). At the completion of the ligation, the DNA ends were blunted with a Klenow polymerase reaction and the blut ends were further ligated to obtain the circular plasmid pBRpt. Again, the annealed DNA was then introduced into *E. coli* (Epicurian E.coli XL-1; Stratagene, Inc.) using a bacterial transformation procedure recommended by Stratagene. Transformation of *E. coli* with pBRpt DNA and screening the transformants for the presence of ~1500 bp insert yielded two clones: BRpt17 and BRpt66. The colony containing the sequences coding for the primary probe was confirmed by analysis of the plasmid DNA. Restriction analysis of the plasmids in these two clones (BRpt17 and BRpt66) confirms that they contain an insert of ~1100 bp, corresponding to about 16 repeatmers in the insert.

The plasmid pBRpt66 was linearized with *Xba* 1 (distal end of T7 promoter) and the riboprobe complementary to the repeatmer insert was prepared in a reaction catalyzed by T7 RNA polymerase in presence of ribonucleotides. The procedure for transcribing DNA plasmid containing the nucleic acid sequence coding for the amplification probe is as follows. Plasmid DNA coding for the amplification probe (10 μg) was treated with the enzyme T-7 RNA polymerase in the method

suggested by the Mega-Transcription kit (Cat. No. 1334) supplied by Ambion Inc. (Austin, Texas). This transcription generated an RNA product with a structure as shown in Figure 1, comprising a poly(U) sequence at the 5' end.

It will be appreciated by one skilled in the art that once the amplification probe is constructed with an appropriate homopolymeric tail, the same template could be used for the production of an amplification probe which could be used in any nucleic acid hybridization assay wherein the promary probe comprises a sequence complementary to the homopolymeric region in the amplification probe.

In general, an *in vitro* transcription of the plasmid coding for the amplification probe using T-7 RNA polymerase would yield its complementary RNA, the amplification probe which carries a poly(U) homopolymeric tail fragment at its 5' end; this poly(U) region is complementary to the poly(A) region of the primary probe described previously. Recent advances in the art allows the large scale production of T-7 polymerase mediated RNA molecules in an *in vitro* transcription. Kits such as "MEGA- Transcript kits" (Ambion Inc., Austin, Texas) could be used to produce milligram quantities of RNA from microgram quantities of the template DNA.

It will be appreciated by one skilled in the art that once the amplification probe is constructed with an appropriate homopolymeric tail, the same template could be used for the production of an amplification probe which could be used in any nucleic acid hybridization assay wherein the primary probe comprises a sequence complementary to the homopolymeric region in the amplification probe.

The Labelling Probe

The immunocaptured probe-target complex can be detected by a variety of well known techniques. In a preferred embodiment, a labelling probe comprising sequences complimentary to at least one of the repeating sequence units on the amplification probe will itself be labelled with a chemical group that is detectable. A detectable chemical group can comprise any material possessing a detectable chemical or physical property. These materials are well known and developed in nucleic acid hybridization assays. Furthermore, most labels useful in such methods can be applied to the present invention. For example, enzymatically active groups have been found to be useful, in particular, those groups that are enzymes (Clin. Chem. (1976)22:1243; U.S. Pat. No. 31,006; and UK Pat 2,019,408), enzyme substrates (U.S. Pat. No. 4,492,751), cofactors (see U.S. Pat. Nos. 4,230,797 and 4,238,565), and enzyme inhibitors (see U.S. Pat. No. 4,134,792). Also useful are fluorescers (see Clin. Chem. (1979)25:353), chromophores, luminescers such as chemiluminescers and bioluminescers (U.S. Pat. No. 4,380,580), as well as specifically bindable ligands such as biotin (European Pat. Spec. 63,879) or a hapten (U.S. Pat. No. 4,380,580), and radioisotopes such as ^3H, ^{14}C, ^{32}P, ^{35}S, and ^{125}I. These labels and labelling pairs are therefore detectable on the basis of either their own physical properties (eg., fluorescers, chromophores and radioisotopes), or their reactive, or binding properties (eg., enzymes, substrates, cofactors and inhibitors). A good example, is a cofactor-labelled antibody that can be detected by addition of the enzyme for which the label is both a cofactor and a substrate for that enzyme. More specifically a hapten or ligand labelled antibody can be detected by adding an antibody to the hapten or a protein (e.g., avidin) which binds the ligand, tagged with a molecule capable of detection. These detectable molecules can be a molecule possessing either a measurable physical property (e.g., fluorescence or absorbance) or a quality capable of participating in an enzyme reaction (see above for list). In one example, one can utilize an enzyme

which acts upon a substrate to generate a product with a measurable physical property. Specific examples of this kind include, but are not limited to, β-galactosidase, alkaline peroxidase and phosphatase. Other similar labelling schemes

5 are evident to one skill in the art.

The nature and the quantity of the label in the labelling probes are not critical. The probes could be labelled either at their extremities or along their entire length with a single or a multiple marker which, of course, can be any

10 detectable substance; in other words, the probe is isotopically or chemically modified in such a way that the person performing the assay can, after further manipulation if necessary, still detect the presence of these labels.

The length of the hybridizing portion of the labelling

15 probe can vary to meet the needs of application of the invention. The minimum length of the probe is about 16 nucleotides and the maximum length is about 25 nucleotides, though a situation may arise where it may be favorable to extend the length beyond 25 nucleotides. In a specific

20 instance, a 25 base polynucleotide fragment with a 5' amino modification was commercially obtained (Bio/Can Scientific Co, Mississauga, Ontario). The sequence in this fragment was complementary to a region within the repeating unit sequence of the amplification probe. The amino modification allowed

25 covalent coupling of the enzyme alkaline phosphatase to the 3' end of the sequence. The covalent attachment of the enzyme to the polynucleotide was carried out according to known methodology (Bio/Can Scientific Co.).

Variations on the design of the labelling probe and the

30 amplification probe may be desirable. For example, the amplification probe itself can be labelled with biotin during the in vitro transcription, or end labelled separately. The labelling probe could be labelled with multiple markers rather than with just a single marker as shown in Figure 1. As long

as the labelling is performed in such a manner as to not interfere with the hybridization between the amplification and labelling probes, these supplementary labels can lead to a proportional increase in sensitivity of the assay

5

This invention describes a general method of constructing the probe cascade. In a general embodiment, the cascade consists of a primary probe, an amplification probe, and a labelling probe with chemical and functional characteristics 10 as described herewithin. The exact sequences implicated in the construction of the probe reagents described above (excepting, of course, the target complementary sequence in the primary probe) are not critical, and only the complementarity of the sequences to be hybridized is 15 important. The exact lengths of the various probes described here are not limited to the sizes given in the examples herein. A person skilled in the art may easily vary these lengths and the methods for achieving such variation are well known in the art.

20 The Assay

The present invention is useful in a large variety of hybridization procedures. The sample to be assayed can be virtually of any medium of interest, such as of medical, veterinary, environmental, nutritional or industrial 25 significance.

One embodiment of this invention comprises a sensitive method of detecting the probe-target complex and its application to the determination of the presence of specific microorganisms in a test sample. An assay, according to the 30 present invention, involves at least the following steps:

a) hybridizing a sequence of a primary polynucleotide probe to a target nucleic acid sequence;
b) immobilizing the probe-target complex;

c)   exposing the immobilized probe-target complex to an
     amplification probe under conditions that allows the
     amplification probe to hybridize to the probe-target
     complex;

5   d)   exposing the hybridized amplification probe to many
          copies of a labelled polynucleotide probe under
          conditions that allows many such labelled probes to
          hybridize to the amplification probe;

     e)   detecting the labelled probes in the resulting

10   complex.

     In one particular embodiment, the sample to be tested is
typically a piece of food, for example meat or cheese, or
another source containing principally double stranded nucleic
acids. This includes microorganisms and/or other cellular

15 material associated with these samples. The test sample is
first treated to release the nucleic acids from the cells.
followed by a denaturation step to denature the nucleic acids.
This is typically accomplished by lysing the cells in a lysis

20 buffer solution and the denaturation of nucleic acids is
preferably accomplished by heating the resulting solution in
boiling water or alkali treatment (e.g., 0.1 N sodium
hydroxide). The denaturing step can often be used
simultaneously as a method to lyse cells. The release of

25 nucleic acids can, also, be obtained through mechanical
disruption such as freezing/thawing, abrasion, sonication,
physical/chemical disruption (eg. polyoxyethylene ether
detergents like Triton®, polyoxytheylenesorbitan detergents
like Tween®, sodium dodecylsulfate, alkali treatment, osmotic

30 shock, heat, or lysing using enzymes such as proteinase K,
lysozyme, pepsin). The resulting medium will contain nucleic
acids in single stranded form which is then assayed according
to present hybridization methods (Wang et al., Appl. Environ.
Microbiol., 1992).

When the sample contains free single-stranded nucleic acid sequences, the sample is in proper form for use with the specific probe. When the assay is performed for detection of a microorganism, a bacterium for example, the cells must be lysed and the nucleic acids have to be exposed in order to be available for hybridization with the probe. Methods of lysis have been previously described and are well known to one skilled in the art.

In one general embodiment, the cells are lysed by mixing 50 μl of the broth culture (about 10^8 to 10^9 cells per ml) with an equal volume of a 2% Triton X-100® and heating at 100°C for 5 minutes in a 1.5 ml eppendorf microfuge tube. The samples are chilled in ice to denature the target nucleic acids. The probe is diluted at a concentration of 200 ng per ml in a buffer containing 8 x SSC (1 x SSC being 0.15 M NaCl plus 0.015 M sodium citrate), 40 mM HEPES (pH 7.4) and 4 mM EDTA. One hundred μl of the probe thus prepared is added to the cell lysate so that the probe is contacted with the target nucleic acid and the hybridization is carried out in this solution at 70°C for 30 minutes.

There are a variety of known hybridization conditions that can be employed in the assay. Typically, hybridization will proceed at slightly elevated temperatures. Typical temperatures range between about 35 and 75°C and are usually about 65°C. The hybridization is carried out in a solution comprised of a buffer at pH between about 6 and 8 and with the appropriate ionic strength. A typical ionic strength is 2 X SCC where 1 X SCC = 0.15 M sodium chloride and 0.015 M sodium citrate at pH 7. The hybridization solution further contains protein such as bovine serum albumin, Ficoll™ (copolymer of sucrose and epichlorohydrin, Pharmacia Fine Chemicals, Piscataway, N.Y.), polyvinylpyrrolidone, and a denatured foreign DNA such as rcm calf thymus or salmon sperm. The degree of complementarity between the target nucleic acid

sequence and the primary probe required for hybridization to occur depends on the stringency of the conditions.

The primary probe is contacted with the denatured nucleic acid sequences in solution phase. The probe-target complexes are removed from the excess unhybridized probe by immunocapture of the former. This is achieved by transferring the hybridization reaction mixture to the wells of a microtiter plate which have been previously coated with monoclonal anti-probe-target complex. The antibody is thus contacted with the probe-target complex present in the hybridization reaction mixture for 30 minutes at 37°C. This contact results in the immunocapture of the probe-target complexes and therefore results in their immobilization to the surface of the wells of the microtiter plate. Any reagent which will subsequently bind to the probe-target complex will thus be immobilized to the solid surface. After the immunocapture reaction is completed, the plates are washed three times with phosphate buffered saline (PBS; 50 mM phosphate buffer containing 0.15 M NaCl) in order to remove the excess unhybridized probes from the well.

Alternatively, any other solid phase could be employed for the immunocapture of the probe-target complexes; any method of immobilisation of the probe-target complex could be used for separating the hybridized probe from the excess unhybridized probe.

In one embodiment, the amplification probe is diluted to a concentration of 200 ng per ml in a buffer containing 4 x SSC, 20 mM HEPES (pH 7.4) and 2 mM EDTA. Two hundred μl of the amplification probe thus prepared are added to the wells of the microtiter plate so that the amplification probe is contacted with the immobilized probe-target complex which carries the poly(dA)$_{12-20}$ at the 3' end of the probe. The plates are incubated at 42°C for 30 minutes so that the hybridization between the poly(dA) of the probe and the

poly(U) of the amplification probe is completed. This poly(dA)-poly(U) is also a DNA-RNA hybrid, thermodynamically the most stable form of a double stranded nucleic acid. At the end of this hybridization period, the plates are washed

5   three times with 0.5 M sodium chloride solution.

The labelling probe is diluted to a concentration of 200 ng per ml containing 4 x SSC, 20 mM HEPES (pH 7.4) and 2 mM EDTA.
Two hundred μl of said labelling probe is added to the

10   microtiter plate wells so that the labelling probes come in contact with the amplification probes which are immobilized to the solid surface. This hybridization is carried out at 42°C for 30 minutes and the plates are then washed three times with 0.5 M sodium chloride.

15   The labelling probe hybridizes with the repeating units of the amplification probe. As the labelling probe is a single stranded polynucleotide, the rate of the hybridization reaction is considerably high.

When the amplification probe comprises sixteen repeating

20   units, sixteen labelling probe molecules can be immobilized per each of the probe-target complexes originally immunocaptured. The signal generated by the probe-target complex is amplified by a magnitude of sixteen times. The enzyme present in the labelling probe is then detected using

25   standard colorimetric, chemiluminescent, fluorometric or other detection methods as discussed herein and is well known in the art.

In one embodiment employing colorimetric methods, a solution containing the substrate to alkaline phosphatase

30   enzyme is added to the wells. For example, 200 μl of p-nitrophenyl phosphate (p-NPP; 4 mg in 10% diethanolamine and 0.5

of incubation at room temperature (20 - 25°C), the color
developed is measured at 410 nm wavelength of light.

Reagent System

Another embodiment of the present invention involves its
5   use in a diagnostic kit, the kit comprising one or more
solutions and devices for carrying out an assay for detection
of food pathogens such as *Listeria monocytogenes* or
Escherichia coli O157:H7 in food samples like cheese or meat.

The present invention further provides for a reagent
10   system. More specifically it provides for a reagent
combination
comprising all of the essential and necessary elements
required to conduct a desired assay method. The reagent
system is presented in a commercially packaged form as a
15   composition or a mixture wherein compatibility of the reagents
will allow for a test device configuration (most typically as
a test kit) a packaged combination of one or more containers,
devices, or the like, holding the necessary reagents and
usually including written instructions describing the
20   performance of the assays. Reagent systems of the present
invention involve all possible configurations and compositions
for performing the various hybridization formats described
herein.

The reagent system will generally comprise bacterial
25   lysis solutions, the solutions containing the target directed
primary probe, the amplification probe, the labelling probe
(preferably labelled with a detectable chemical group) enzyme
substrates, microtiter plates or strips coated with the anti-
target-probe complex antibodies, and a description of the
30   assay comprehensible to one skilled in the art. A test kit
form of the reagent system may further include ancillary
chemicals. Such ancilliary chemicals can include components
of the hybridization solution and denaturation agents capable

of converting double stranded nucleic acids found in a test
sample into single stranded form. More preferably, there is
included a chemical lysing and denaturing agent, such as an
alkali, for treating the sample in order to release single
5   stranded nucleic acid therefrom.

The present invention will now be illustrated, but is not
intended to be limited, by the following example.

Example 1:   Assay for the detection of L. monocytogenes in
cheese.

10   Twenty five grams of the test cheese was homogenized for
2 minutes in a stomacher with 250 ml of 1% sterile peptone
water and allowed to settle for about 15 minutes. A 0.5 ml
sample of the clear upper portion of this homogenate was
inoculated into 10 ml of trypticase soy -0.6% yeast extract
15   broth and incubated at 37°C for 16 - 20 hours.

Any bacteria in 100 μl of the enriched broth was lysed
by mixing the broth with an equal volume of 2% Triton X-100 in
a microfuge tube and boiling for 5 minutes at 100°C. The
solution was quickly chilled in ice.

20   Probe solution (100 μl; 200 ng per ml) in 8 x SSC, 40 mM
HEPES (pH 7.4) and 4 mM EDTA was added to the lysate and
incubated at 37°C for 30 minutes. The probe selected here was
DNA in nature and specifically hybridising with the ribosomal
RNA (rRNA) of L. monocytogenes. The hybridization reaction
25   mixture (200 μl) was transferred to a microtiter plate well
which had been previously coated with monoclonal anti-probe-
target antibodies and incubated at 37°C for 30 minutes. The
plates were then washed 3 times with PBS.

The amplification probe solution (200 μl; 200 ng/μl) in 4
30   x SSC, 20 mM HEPES (pH 7 4) and 2 mM EDTA was added to the

wells and incubated at 37°C for 30 minutes. The plates were
then washed 3 times with 0.5 M sodium chloride solution. The
labelling-probe solution (200 μl, 200 ng/μl) in 4 x SSC, 20 mM
HEPES (pH 7.4) and 2 mM EDTA was added to the wells and
5 incubated at 37°C for 30 minutes. The plates were washed 3
times with 0.5 M sodium chloride solution. Alkaline
phosphatase substrate solution (4 mg of p-NPP in 10%
diethanolamine and 0.5 mM magnesium chloride, pH 9.8) was
added to the wells and incubated at room temperature for 15 to
10 30 minutes and the color developed was measured at 410 nm in a
standard microtiter plate reader.

With the help of positive and negative controls, a
colorimetric value was chosen that was statistically distinct
between samples that contain and those that do not contain L.
15 monocytogenes. Using this "cut-off value", the color
generated was interpreted as to whether the original sample
was positive or negative for the presence of the pathogen.

Below are results of assays performed that detect the
presence of L. monocytogenes. Assay #1 was performed with
20 biotinylated probe which is 784-bp long; the hybridized probe
was detected with a streptavidin-alkaline phosphatase
conjugate system. Assay #2 was performed according to the
protocol given herein.

Sample	Status	LM count (L.m./ml enriched broth)	O.D.	
			Assay #1	Assay #2
1	Positive Control	5.0×10^3	0.20	1.85
2	Positive Control	3.0×10^3	0.19	1.93
3	Positive Control	7.0×10^6	0.43	2.99

Sample	Status	LPM count (L.m./ml enriched broth)	O.D. Assay #1	Assay #2
4	Positive Control	5.0×10^6	0.31	2.45
5	Positive Control	6.0×10^5	0.24	2.42
6	Positive Control	7.0×10^4	0.19	2.23
7	Negative Control	0	0.00	0.07
8	Negative Control	0	0.18	0.16
9	Negative Control	< 10	0.09	0.27
10	Negative Control	< 10	0.00	0.03
11	Negative Control	0	0.02	0.21
12	Negative Control	0	0.04	0.32
13	Unknown	< 25	0.00	0.09
14	Unknown	5×10^6	0.22	2.41
15	Unknown	9×10^4	0.32	2.27
16	Unknown	7×10^2	0.23	1.87
17	Unknown	5×10^2	0.16	1.64
18	Unknown	5×10^6	0.27	2.46
19	Unknown	1×10^5	0.21	2.12
20	Unknown	2×10^3	0.13	1.56

It is to be understood that the examples described above are not meant to limit the scope of the present invention. It is expected that numerous variants will be obvious to the person skilled in the art to which the present invention

pertains, without any departure from the spirit of the present invention. The appended claims, properly construed, form the only limitation upon the scope of the present invention.